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The displacing effect of a fatty acid on the binding of diazepam to human serum albumin

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Diazepam is a drug used very widely in the treatment of anxiety [1]. Studies on protein binding of diazepam in dog plasma and bovine serum showed extensive binding of the drug [2]. However, studies with human plasma or serum have been neglected. Drugs which are bound extensively to proteins present enhanced possibilities of interaction between drugs and endogenous substances [3]. This study was conducted to investigate the binding characteristics of diazepam to human serum albumin (HSA) and the effect of a fatty acid on the drug-protein binding.

Crystalline human serum albumin, essentially free of fatty acids, and sodium laurate were obtained from Sigma Chemical Co. 5-[^{14}C]Diazepam (sp. act., 197 μCi/mg) was supplied by Hoffman-La Roche Inc. The radiochemical purity of 5-[^{14}C]diazepam verified by thin-layer chromatography was found to be greater than 98 per cent [4]. Radioactive drugs were dissolved in absolute ethanol and mixed with unlabeled drug to achieve suitable concentrations. Unless otherwise indicated, 0·5% (w/v), $7{\cdot}46 \times 10^{-5}$ M, HSA was used and dissolved in 0·1 M phosphate buffer of pH 7·4. The drug solution (0·1 ml) was added to 4·9 ml HSA–buffer solution and stirred in a vortex mixer for 1 min. In some experiments, sodium laurate was added to the HSA–buffer solution and stirred for 1 min before adding diazepam. Diazepam concentrations ranged from $3{\cdot}6 \times 10^{-6}$ M to $1{\cdot}01 \times 10^{-4}$ M and laurate was $3{\cdot}5 \times 10^{-4}$ M. The molar ratios of laurate/diazepam ranged from 970 to 3·5. The molar ratio of laurate/albumin was 4·7 in 0·5% HSA. All experiments were carried out at the controlled room temperature of 22°.

Membrane cones (Centrifro CF-50 membrane ultrafilter, Amicon Corp.) were soaked in distilled water for at least 1 hr before use. After removing the water on the membrane by centrifugation, 4 ml of drug–HSA solution was pipetted into the cone, and centrifuged twice at 1000 rev/min for 2·5 min. After each centrifugation, approximately 0·1 ml of the ultrafiltrate was removed for radioactivity counting; the exact volume of ultrafiltrate was measured by weighing. Drug containing albumin solution (0·1 ml) from inside the cone was also taken for radioactivity counting. The radioactivity was determined using Aquasol (New England Nuclear) and Nuclear Chicago Mark II liquid scintillation counter. The amount of radioactivity in the ultrafiltrate was 1,500–10,000 dis./min/ml, and the standard deviation among repetitive determinations of radioactivity ranged from 1 to 3 per cent. Potentially major sources of error were protein leakage through the membrane and adsorptive binding of the drug to the membrane cone. These factors were carefully examined under these conditions. A small amount of albumin leaked through the membrane if the filtrate volume exceeded 0·2 ml. The quantity of drug adsorbed to the cone was determined by subtracting the arithmetic sum of the filtrate and supernatant drug from the original quantity of drug–albumin solution in the cone. Albumin concentrations in the ultrafiltrate were measured by the biuret method [5]. The percentage of drug bound to albumin was calculated by deducting the free drug concentration in the filtrate from the original concentration (free plus bound) in the supernatant. The calculated free drug concentration included the quantity of drug adsorbed to the membrane cone.

The binding capacity of diazepam was studied at a concentration observed to occur in therapy, that is, a concentration of 248 ng/ml with 3·5% HSA in buffer. Diazepam binding to HSA was 93·7 ± 0·8 (S.E.) per cent. Van der Kleijn [2] reported that the extents of binding of diazepam to dog plasma protein and bovine serum albumin (BSA) were 93–94 per cent and 84–87 per cent, respectively, as determined by an ultracentrifuge method. Per cent binding of diazepam to dog plasma and to BSA appeared to be independent of the drug concentration over a range

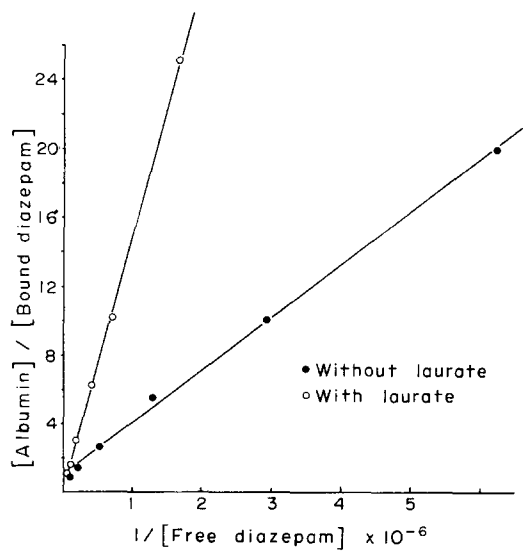

Fig. 1. Binding of diazepam to human serum albumin and the inhibiting effect of laurate. Albumin 0·5% (w/v), $7{\cdot}46 \times 10^{-5}$ M; laurate, $3{\cdot}5 \times 10^{-4}$ M. Both ordinates are calculated on the basis of molarities.

of therapeutic concentrations [2]. In the present study, binding was shown to be concentration dependent over the range of relatively high concentrations, 0·29 to 1·01 × 10^{-4} M, but was independent over the range near therapeutic concentrations.

To obtain binding parameters, 0·5% HSA and diazepam concentrations ranging from 3·6 × 10^{-6} M to 1·01 × 10^{-4} M were employed. The low concentration of 0·5% HSA was found to be appropriate for these studies because of the low solubility of diazepam in aqueous solutions. The displacement of diazepam by laurate tended to increase with decreasing concentration of diazepam. At therapeutic concentrations, diazepam was displaced as much as 13–14 per cent. At the highest concentration (1·01 × 10^{-4} M) which was well above the therapeutic concentrations, the influence of laurate became insignificant.

Figure 1 shows the double-reciprocal plot [3] which clarifies the nature of the displacement of diazepam from albumin by a fatty acid. For the data obtained in the absence and in the presence of laurate, the slopes of the straight lines have the values of 3·3 × 10^{-6} and 1·5 × 10^{-5} M respectively. The ordinate intercept appears common to both lines, suggesting that the inhibition is competitive. However, close inspection of the graph shows that the estimate of the magnitude of the ordinate intercept is less certain than is the estimate of slope; the intercept has a value of 1 or less, allowing the conclusion that each albumin molecule has one or more binding sites for diazepam. Therefore, the slope of 3·3 × 10^{-6} M must be interpreted as indicating the lower limit of the dissociation constant of an albumin–diazepam complex. Mueller and Wollert [6] investigated the binding of eleven benzadiazepines to HSA by means of the gel filtration technique and reported that HSA has a single binding site for diazepam with a dissociation constant of about 2·2 × 10^{-6} M. This value is comparable to the data presented here.

If the albumin binding of a drug is extensive, interactions with other drugs or substances should be considered. Free fatty acids are physiological substances which have high affinity to serum albumin, and there are numerous studies concerning interactions between fatty acids and drugs [7, 8]. Lauric acid (8·8 × 10^{-4} M) has been shown to inhibit the binding of phenylbutazone and warfarin to human albumin [8]. In the present study, at the molar ratio of 4·7 for laurate to albumin, this fatty acid showed a significant inhibitory effect on the binding of diazepam to human serum albumin. This molar ratio of 4·7 was smaller than that reported by Rudman *et al.* [7] who demonstrated that the binding of salicylates or other ligands was inhibited by free fatty acids at a molar ratio of 7.

The binding of drugs to plasma protein or tissue can influence the distribution and elimination of drugs [9]. On the other hand, protein binding itself can be modified by numerous factors such as protein concentrations, dehydration, alteration of pH or the presence of competing substances [3]. Our experiments *in vitro* showed a displacement of diazepam by a free fatty acid at plasma concentrations encountered during therapeutic use of diazepam. Therefore, the possibility exists that an interaction between diazepam and free fatty acids could be observed clinically in man. It has been observed that plasma concentrations of diazepam fluctuate after a single dose in man and it has been suggested that this could be due to an enterohepatic circulation [10]. However, it could equally well be hypothesized that the fluctuations of diazepam in blood result from variations in plasma free fatty acids with a consequent change in diazepam binding to albumin and thus in the distribution of diazepam.

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